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Saccharomyces cerevisiae C1D is implicated in both non-homologous DNA end joining and homologous recombination

Tuba Erdemir,[1] Bilada Bilican,[2] Tolga Cagatay,[1] Colin R. Goding[2†] and Ugur Yavuzer[1]*
[1]*Bilkent University, Molecular Biology and Genetics Department, 06533 Bilkent, Ankara, Turkey.*
[2]*Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK.*

Summary

C1D is a gamma-irradiation inducible nuclear matrix protein that interacts with and activates the DNA-dependent protein kinase (DNA-PK) that is essential for the repair of the DNA double-strand breaks and V(D)J recombination. Recently, it was demonstrated that C1D can also interact with TRAX and prevent the association of TRAX with Translin, a factor known to bind DNA break-point junctions, and that over expression of C1D can induce p53-dependent apoptosis. Taken together, these findings suggest that mammalian C1D could be involved in maintenance of genome integrity by regulating the activity of proteins involved in DNA repair and recombination. To obtain direct evidence for the biological function of C1D that we show is highly conserved between diverse species, we have analysed the *Saccharomyces cerevisiae* C1D homologue. We report that the disruption of the *YC1D* gene results in a temperature sensitivity and that *yc1d* mutant strains exhibit defects in non-homologous DNA end joining (NHEJ) and accurate DNA repair. In addition, using a novel plasmid-based *in vivo* recombination assay, we show that *yc1d* mutant strains are also defective in homologous recombination. These results indicate that *YC1D* is implicated in both homologous recombination and NHEJ pathways for the repair of DNA double-strand breaks.

Introduction

Chromosomes occupy specific nuclear domains and most of the chromatin is further organized into looped domains by the binding of certain DNA regions to a network of intranuclear proteins, termed the nuclear matrix. Recent evidence suggests that the nuclear matrix plays essential roles in important cellular processes, such as recombination, DNA repair, transcription regulation, translocation and apoptosis (Bode *et al*., 2000). The recently identified nuclear matrix protein C1D belongs to the family of non-histone polypeptides involved in higher order chromatin folding (Nehls *et al*., 1998). Although the biological functions for these proteins are still largely obscure, some have been reported to be associated with highly repetitive DNA sequences and involved in targeting a subset of genomic DNA to the nuclear matrix (Neuer and Werner, 1985; Neuer-Nitsche *et al*., 1988; Werner and Neuer-Nitsche, 1989).

Accumulating evidence suggests that C1D may play an important role. C1D interacts with and activates DNA-dependent protein kinase (DNA-PK) (Yavuzer *et al*., 1998), which plays a key role in DNA double-strand break (DSB) repair and in V(D)J recombination, a process specific to lymphocytes that is required for development of the immune system (Smith and Jackson, 1999). Moreover, the mRNA and protein levels of C1D are induced in response to DNA damaging agents specifically causing double-strand DNA breaks. These findings raised the possibility that C1D may play a role in DSB repair by recruiting and linking the DNA-PK function to the nuclear matrix. In support of this observation, the *xrs5* cells deficient in one of the subunits of DNA-PK, Ku-80, exhibit irregularly shaped nuclear envelope and altered nuclear matrix compared with their wild-type controls (Yasui *et al*., 1991; Korte and Yasui, 1993).

In addition, overexpression of C1D can induce apoptosis in a p53-dependent manner in tissue culture cells (Rothbarth *et al*., 1999). However, the molecular mechanisms underlying the C1D-induced apoptosis are not well defined.

More recently, we used the two-hybrid system to identify TRAX (translin-associated protein X), as a C1D-interacting protein (Erdemir *et al*., 2002). Significantly, the C1D/TRAX interaction is induced specifically in response to γ-irradiation in mammalian cells, again consistent with a role for C1D in the DNA-damage response. TRAX binds

Accepted 19 August, 2002. *For correspondence. E-mail ugur.yavuzer@itu.edu.tr; Tel. (+90) 212 285 3301; Fax (+90) 212 285 6386. †Present address: Molecular Biology and Genetics Department, Istanbul Technical University, 80126 Maslak, Istanbul, Turkey.

strongly to the DNA/RNA binding protein translin (Aoki *et al*., 1997) and is thought to be the regulator of translin, which recognizes a consensus DNA sequence found at the breakpoint junctions of certain chromosomal translocations seen in some types of lymphoid malignancies (Aoki *et al*., 1995) and solid tumours (Chalk *et al*., 1997). The TRAX–translin complex has a high affinity towards DNA and TRAX has been shown to enhance the DNA binding capacity of translin, while decreasing its RNA-binding ability (Chennathukuzhi *et al*., 2001). Intriguingly, γ-irradiation induced the formation of a stable C1D–TRAX complex and prevented the association of TRAX with translin complex, suggesting that C1D could prevent binding of translin–TRAX to DNA and, as a consequence, may inhibit any unwanted recombination events when DNA is damaged. Taken together, the data suggest that C1D is a multifunctional protein and may be capable of regulating a range of cellular events, such as DNA repair, recombination and apoptosis. Deciphering the molecular mechanisms C1D uses while performing its functions is clearly a key issue.

Here we show that C1D appears to represent a member of a highly conserved family of proteins present in organisms as diverse as yeast, flies, plants and mammals. Given that *Saccharomyces cerevisiae* has served as a useful model organism for analysing mammalian gene function and deducing the biological function of key mammalian proteins, we chose to examine the potential role of the *S. cerevisiae* homologue of C1D, that we term *YC1D*, in DNA repair and recombination. Deletion of the *YC1D* gene results in a temperature-sensitive phenotype. Using a plasmid-based *in vivo* DSB repair assay, we demonstrate that *yc1d* mutant strain exhibits defects in both non-homologous end joining (NHEJ) and homologous recombination (HR), the two main pathways used to repair DNA double-strand breaks.

Results

Identification of YC1D

Using a BLASTP search, we found several proteins from a number of species (*Caenorhabditis elegans, Arabidopsis thaliana, Drosophilia melanogaster, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) sharing a significant homology with human and mouse C1D (Fig. 1). The overall similarity is 25%–37% but the homology is particularly strong across a central core of the protein where the entire family posses 50% sequence identity. Moreover, again within this central region, prolines and glycines (indicated in green) that may mark the boundaries of alpha helices and beta sheets are highly conserved amongst all the species examined. The high degree of conservation between species suggests that the C1D family of proteins is likely to be involved in important biological processes.

Phenotypic consequences of YC1D *deletion*

In an attempt to identify a function for C1D, we took advantage of the genetic approach possible using the yeast system and examined the phenotypic consequences of inactivating the gene encoding the *S. cerevisiae* homologue of the mammalian C1D proteins that we term *YC1D* in both haploid and diploid yeast strains.

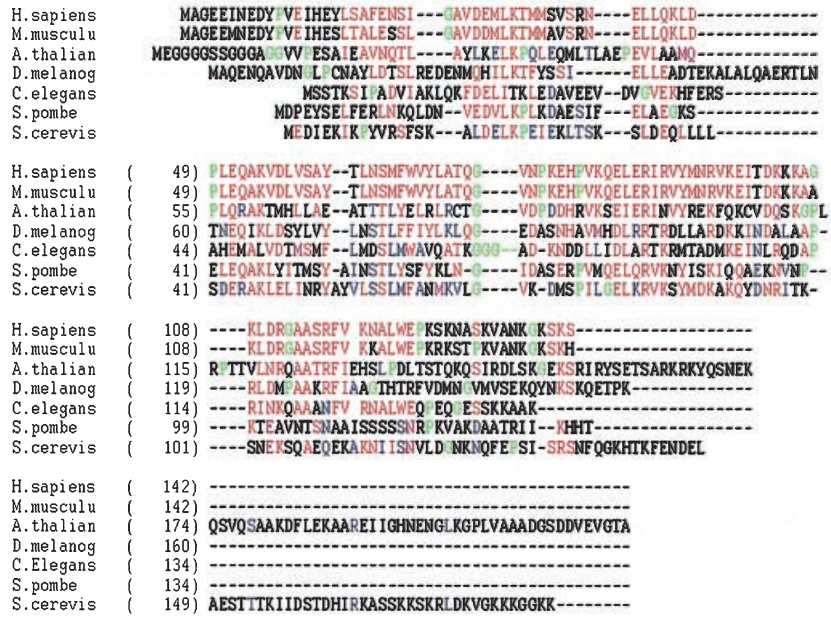

Fig. 1. C1D is evolutionarily conserved. Alignment of C1D from *Homo sapiens* (NM_006333), *Mus musculus* (NM_020558), *Drosophilia melanogaster* (AE003500), *Caenorhabditis elegans* (NM_061564), *Saccharomyces cerevisiae* (NC_001140), *Schizosaccharomyces pombe* (NC_003421) and *Arabidopsis thaliana* (NM_122417). The amino acid position in each protein is shown on the left. Prolines and glycines that may mark the boundaries of alpha helices and beta sheets are indicated in green. Those residues with strong homology or identity to the human and mouse sequences are indicated in red, whereas those that are not homologous in human and mouse but show homology with the *S. cerevisiae* sequence are in blue.

In an initial screen for a phenotype associated with the loss of the *YC1D* gene, we noted that *MATa*- and *MATα*-type haploid and diploid strains deleted for the YC1D gene were temperature-sensitive for growth at 39°C (Fig. 2). On the contrary, the heterozygous diploid strain, *YC1D/yc1d*, did not show any defect for growth at the restricted temperature, suggesting that the temperature-sensitive phenotype is a direct consequence of Yc1dp deficiency.

Given the possible role of the mammalian C1D protein in DNA repair, we next examined the effect of *YC1D* disruption on the sensitivity of yeast strains towards various DNA damaging agents. The *yku70, rad52* and *mec1–21* mutant strains that have previously characterized defects in DNA damage repair were also included as controls. Similar to the *yku70* mutant strain (Boulton and Jackson, 1996b), the *yc1d* mutant haploid strains did not exhibit sensitivity towards agents such as ultraviolet light (UV) or methyl methane sulphonate (MMS) (Fig. 3A and B). However, both the *MATa*- and *MATα*-type *yc1d* haploid mutant strains were slightly sensitive towards γ-irradiation (Fig. 3C). As expected and reported before, the *rad52* mutant strain was sensitive to MMS, UV-, and γ-irradiation, whereas the *mec1–21* mutant strain was sensitive to UV irradiation. Previously, it was demonstrated that inactivation of *YKU70* hypersensitizes *rad52* mutant strains to γ-irradiation (Boulton and Jackson, 1996b). However, no increased γ-sensitivity was detected in strains mutant for both *YKU70* and *YC1D* (data not shown).

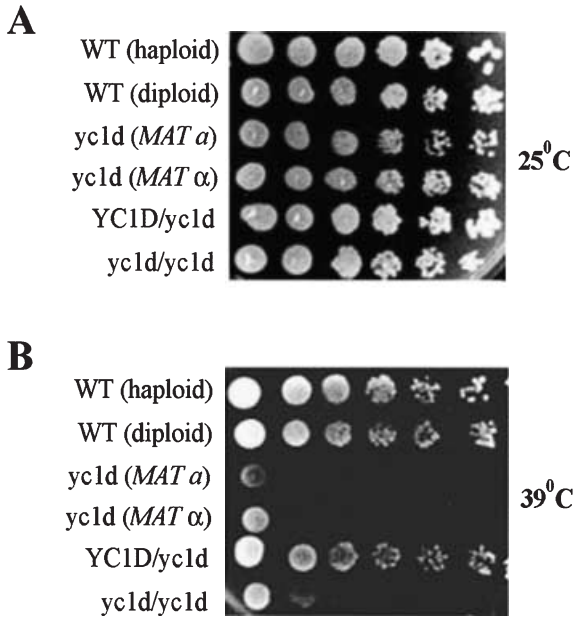

Fig. 2. Deletion of YC1D renders cells sensitive to high temperatures. Wild-type (WT) haploid and diploid strains, *MATa*- and *MATα*-type haploid *yc1d* mutant strains and heterozygote (*YC1D/yc1d*) and homozygote (*yc1d/yc1d*) diploid mutant strains were spotted on YPAD plates in serial 10-fold dilutions and incubated at either 25°C (A) or 39°C (B) for 3 days.

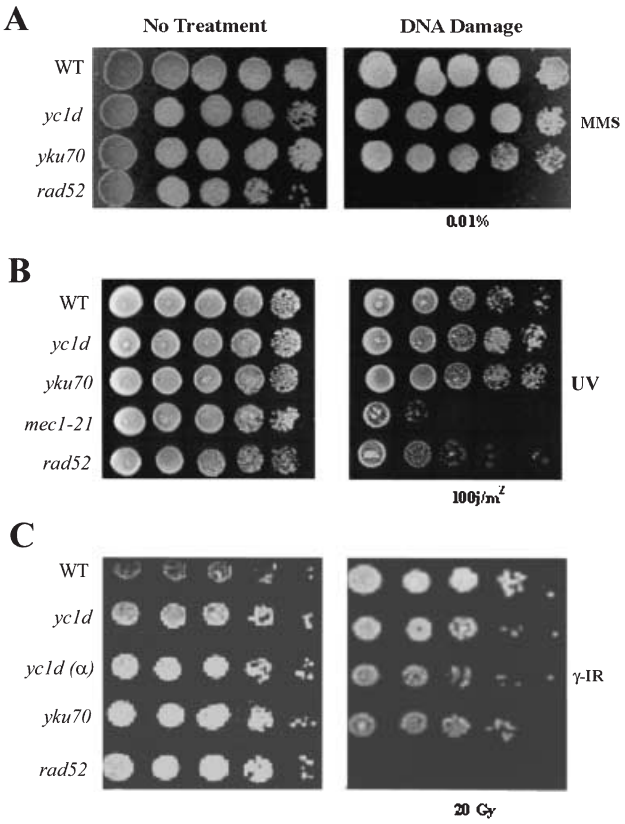

Fig. 3. The *yc1d* mutant strains are slightly sensitive towards γ-irradiation. A single colony from wild-type (WT), *yc1d, yc1d* (*MATα), yku70*, *rad52 and mec1–21* mutant strains were grown to mid-log phase and aliquots (5 μl) of serial 10-fold dilutions of yeast cultures were spotted onto YPAD plates containing 0.01% MMS (A) or exposed to 100 j/m^2 of UV irradiation (B) or 20 Gy γ-irradiation (C). The plates were then incubated at 30°C for 3 days.

Yc1d *mutants are defective in DSB repair*

In *S. cerevisiae*, DNA DSBs are repaired mainly by homologous recombination. However, non-homologous end-joining can also be detected in the absence of proteins catalysing homologous recombination or in the absence of homology between linear plasmid molecules and the host genome (Boulton and Jackson, 1996a,b; Siede *et al.*, 1996). A plasmid-based repair assay was used to assess the efficiency of DNA DSB repair (Boulton and Jackson, 1996b). A *S. cerevisiae–E. coli* shuttle plasmid was linearized within its multiple cloning site using restriction enzymes that produce 5′- and 3′- overhanging or blunt-ends and the linearized and supercoiled versions of the same plasmid were then introduced into the wild-type, *yc1d* and *yku70* mutant strains in parallel. Linearized plasmids can only be propagated in *S. cerevisiae* after they have been re-circularized and ligated. Therefore, by counting the number of colonies harbouring re-ligated plasmid on each plate and normalizing the potential minor

differences in transformation efficiencies between various yeast strains with the supercoiled version of the same plasmid, the efficiency of ligation, thus NHEJ, can be measured. As seen in Fig. 4A, in the wild-type strain, both the 5′- and 3′-overhanging ends were re-ligated efficiently, whereas as reported previously, the plasmids bearing two blunt termini (as generated by *Sma*I digest) were not (Boulton and Jackson, 1996b). We observed that the *yc1d* mutant strain was defective in rejoining the 3′-overhanging (*Sac*I and *Pst*I digests) and blunt-ended (*Sma*I digest) plasmid DNA, however, intriguingly unlike the *yku70* mutant strain, the *yc1d* mutant strain exhibited only a slight defect in repair of the 5′-overhanging ends (Fig. 4A). To confirm that this slight defect seen in the *yc1d* mutant strain is not due to the plasmid or the restriction enzyme used, we analysed a different plasmid, pGV255-live, and linearized this plasmid with a different enzyme, *Xho*I, to produce 5′-overhanging ends and also with *Sac*I or *Sma*I to produce 3′-overhanging and blunt termini respectively. It was observed that, similar to the previous results, the *yc1d* mutant strain exhibited a slight defect in re-joining the 5′-overhanging ends and was defective in both 3′-overhanging and blunt-ended plasmid repair (Fig. 4B). These data suggest that, like Yku70p, Yc1dp may also be involved in NHEJ. Indeed, when a plasmid expressing wild-type Yc1dp was introduced into the *yc1d* mutant strain (yc1d/pWT C1D), the defect in efficient 3′-end-joining in the *yc1d* mutant strain was restored to the wild-type levels (Fig. 4C). As deletion of *YC1D* or *YKU70* genes results in inefficient joining of 3′-overhanging ends, we assayed the double knockout strains mutant for *YC1D* and *YKU70* genes in end-to-end joining of a *Sac*I-linearized

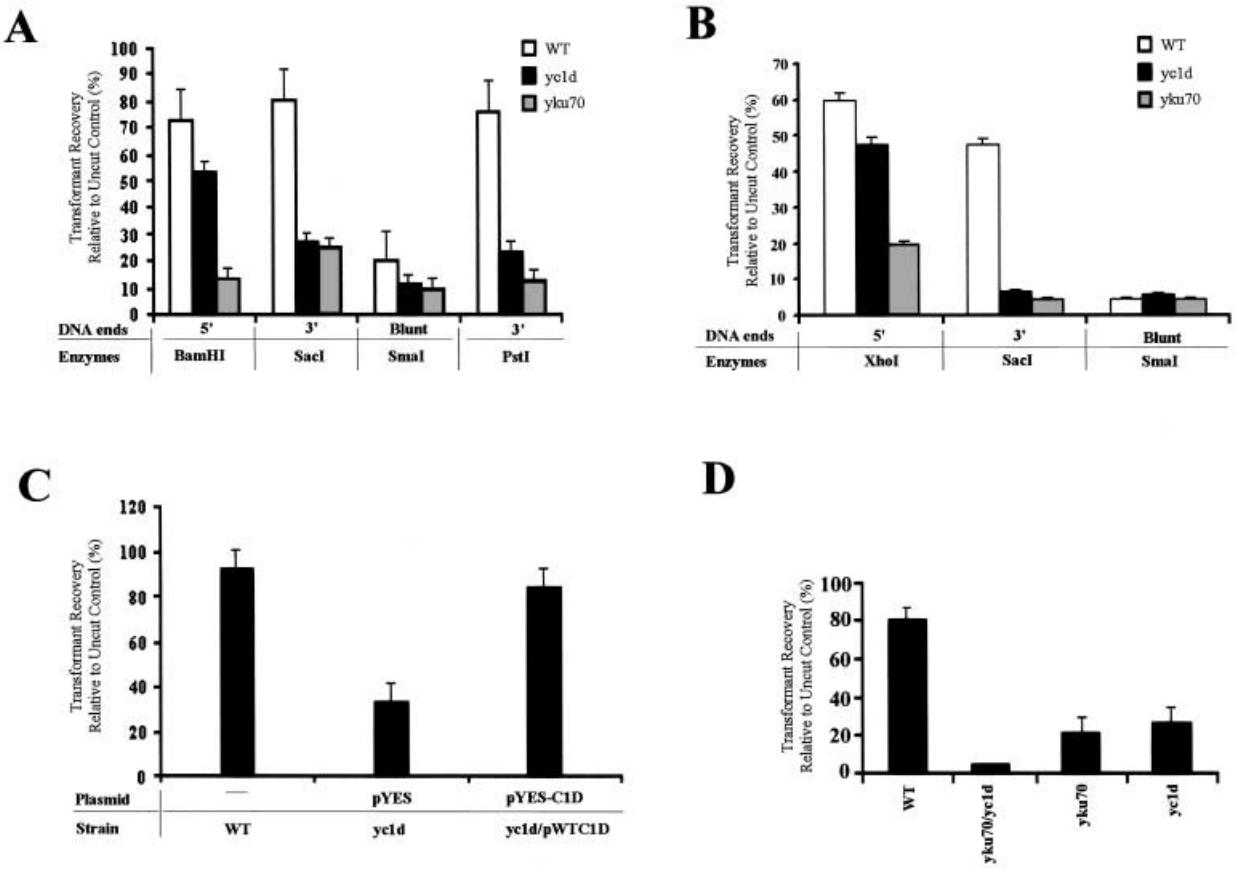

Fig. 4. The *yc1d* mutant strain is defective in NHEJ.
A. The yeast episomal plasmid, pRS313, was linearized using *Bam*HI, *Sac*I, *Sma*I or *Pst*I and transformed into the indicated yeast strains in parallel with the supercoiled version of the plasmid. The colonies formed were counted and normalized to the transformation efficiency for each strain.
B. A different plasmid, pGV255-live was linearized using *Xho*I, *Sac*I or *Sma*I and analysed as in (A).
C. The plasmid repair defect of *yc1d* mutant strain is complemented by a plasmid expressing wild-type Yc1dp. The indicated strains either did not contain a plasmid or contained the plasmid pYES-C1D (pWT/C1D) or parental plasmid (pYES). The *Sac*I-linearized pRS313 was transformed into these strains in parallel with the supercoiled version of the plasmid, and the transformants yielded were plotted as described before.
D. A *yc1d/yku70* double mutant strain is more defective in re-ligation of 3′-overhanging DNA ends with respect to the *yc1d* or *yku70* mutant strains. The *Sac*I-linearized pRS313 was transformed into the indicated yeast strains and analysed as described above.

plasmid. As seen in Fig. 4D, a double knockout mutant strain exhibited a further reduction in the efficiency of repair of the 3′-overhanging DNA ends when compared with *yc1d* or *yku70* mutant strains.

In the absence of YC1D *accurate end joining is impaired*

Although the *yc1d* mutant strain is deficient in repair of 3′-overhanging ends, there is a significant level of residual repair activity (Fig. 4A). It was important therefore, to analyse the transformants and determine whether the NHEJ has been performed accurately in the absence of *YC1D.* For this purpose, the yeast episomal plasmid pGV255-live, expressing *lacZ* constitutively, was used. When this plasmid is introduced into yeast, the colonies produce a blue colour in the presence of the substrate Xgal, due to the expression of β-galactosidase. The pGV255-live was linearized using the unique *Sac*I site that is found in the middle of the LacZ open reading frame (ORF), introduced into the wild-type, *yc1d* and *yku70* mutant strains and plated onto selective medium. As described previously, only the yeast cells harbouring the re-circularized and ligated plasmids can be propagated and grow on selective medium. In this case, because the plasmid is linearized within the coding sequence of LacZ, re-ligation will produce transformants, but only the colonies harbouring accurately re-ligated plasmid will give a blue colour in the presence of Xgal. To confirm that the plasmids isolated from the blue colonies have been re-ligated accurately, we digested the isolated plasmids with *Sac*I and demonstrated that the plasmids were linearized, thus did not lose the *Sac*I recognition site (data not shown). Therefore, the ratio of the number of blue colonies to the total number of transformants was taken as the efficiency of accurate repair.

The result showed that the *yc1d* mutant strain is defective in accurate repair of DNA ends, as only 50% of the transformants harbouring the re-ligated plasmid were accurately repaired (15% out of 30% of the residual repair activity), thus giving blue colour in the presence of Xgal (Fig. 5). In the remaining transformants, although the plasmid was repaired, colonies did not produce blue colour indicating an inaccurate repair. In agreement with a previous report, absence of Yku70p leads to error-prone repair of DNA ends (Boulton and Jackson, 1996b) and only 25% of the transformants were repaired accurately. As the wild-type strain results in accurate DNA repair, these data point to Yc1dp acting as a barrier to error-prone DNA repair pathways.

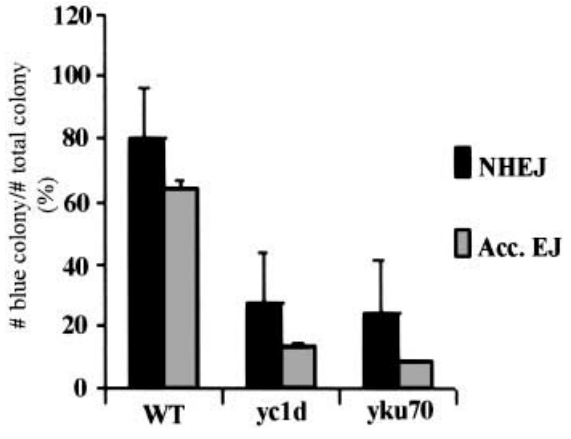

Fig. 5. The strains deficient for YC1D cannot perform DNA end-joining accurately. The yeast episomal plasmid, pGV255-live, was linearized using *Sac*I (producing 3-overhanging ends) within the coding region of *lacZ* and transformed into the indicated yeast strains. Colony-lift β-galactosidase assay was performed on the transformants and the ratio of the number of blue colonies to the number of total colonies was calculated.

Role of YC1D *in homologous recombination*

As the repair of DSBs may also be performed by homologous recombination, the role of *YC1D* gene in recombination was assayed. For this purpose, a novel *in vivo* recombination assay was developed using the pGV255-live plasmid that expresses *lacZ* under the control of the constitutively active *CYC* (cytochrome C) promoter. As shown schematically in Fig. 6, when this plasmid is introduced into yeast, the colonies produce a blue colour in the presence of the substrate Xgal due to the expression of β-galactosidase (Fig. 6A). The pGV255-live was modified by inserting a double-stranded oligonucleotide into the unique *Sac*I site within the LacZ coding sequences. This 21 bp oligonucleotide contains a unique restriction enzyme site (*Bgl*II) flanked by 'STOP' codons in different reading frames, thus disrupting the expression of *lacZ*. We termed this plasmid 'pGV256-dead' as no β-galactosidase activity is produced by the yeast transformants harbouring this plasmid (Fig. 6B). When the pGV256-dead is linearized from the unique *Bgl*II site and introduced into the recipient yeast strain, the re-circularized and ligated plasmids can be propagated in yeast yielding transformants that do not produce blue colour in the presence of the substrate (Fig. 6C). For the homologous recombination assay, the *Bgl*II-linearized pGV256-dead plasmid was introduced into the recipient yeast strains together with a PCR product encompassing the region of LacZ where STOP codons were integrated. As there is no homology between the LacZ fragment and *S. cerevisiae* chromosomal sequences, the only way the plasmid can be propagated is either by re-circularization and ligation of the plasmid, in which case there would be transformants, but none of them will produce blue colour in the presence of Xgal. Alternatively, the 800 bp LacZ PCR fragment could be integrated into the plasmid by homologous recombina-

tion, which will manifest itself by production of blue colour as a consequence of the reconstitution of *lacZ* expression (Fig. 6D). Therefore, the ratio of blue colonies over to the total number of colonies on a selective plate will directly reflect the efficiency of homologous recombination for those particular yeast strains.

To validate this novel *in vivo* recombination assay, the *rad52* mutant strain that is deficient in HR (Paques and Haber, 1999) and the *yku70* mutant strain, which is mainly deficient in DNA end joining but not in HR (Lewis *et al*., 1999), were also analysed along with the wild-type and *yc1d* mutant strains. Using this assay, it was demonstrated that the *yku70* mutant strain was able to perform HR at least as efficiently as the wild-type strain, whereas the *rad52* mutant strain was completely defective in HR in accordance with the previous findings (Fig. 7A). Interestingly, the *yc1d* mutant strain exhibited a twofold reduction in HR compared with the wild-type strain. Note that the average of total number of colonies from four separate experiments was: 705 colonies (423 white and 282 blue) for the wild-type strain and 430 (335 white and 95 blue) and 125 colonies (65 white and 60 blue) for the *yc1d* and *yku70* mutant strains respectively. When the total number of colonies was normalized to the number of colonies obtained by the uncut plasmid for each yeast strain, it was seen that the percentage of plasmid repair efficiency between each strain was similar to the previous results. However, as *S. cerevisiae* prefers HR to repair DNA double-strand breaks, and because in this assay this opportunity is given to the yeast cells, the plasmid repair was performed by HR as well as NHEJ. Indeed, almost 50% of the total colonies in the *yku70* mutant strain, for example, were blue, suggesting that the plasmid repair was performed by both HR and NHEJ.

To confirm the results obtained using this LacZ-based *in vivo* recombination assay, we also checked the same strains for growth in the presence of the homothallic switching (HO) endonuclease by using a galactose-inducible construct. It has previously been shown that strains deficient in homologous recombination cannot survive under the conditions when HO endonuclease is expressed if they harbour this particular construct (Lewis *et al*., 1999). Therefore, we transformed WT, *rad52* and *yc1d* mutant strains with a plasmid expressing HO endonuclease under a galactose-inducible promoter and assessed the survival rates by counting the number of colonies formed on galactose-containing medium. As seen in Fig. 7B, survival of *rad52* mutant strain was

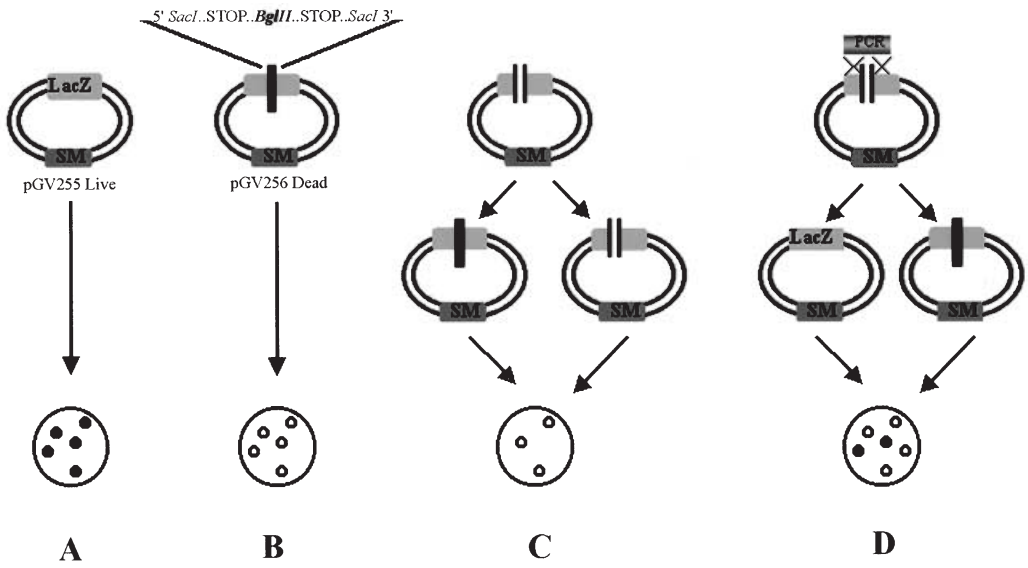

Fig. 6. Schematic drawing of *in vivo* recombination assay.
A. The yeast episomal plasmid pGV255-live expresses *lacZ* constitutively and yeast colonies harbouring this plasmid produce blue colour in the presence of the substrate Xgal (denoted as closed circles).
B. The pGV255-live has been modified by inserting a double-stranded oligonucleotide carrying STOP codons into the LacZ coding sequences. The resulting plasmid, 'pGV256-dead' can be propagated in yeast, however, the colonies do not produce blue colour in the presence of Xgal (open circles).
C. When the pGV256-dead is linearized and transformed into yeast cells, due to non-homologous DNA end joining (NHEJ), a certain proportion of the linearized plasmid will be re-ligated and propagated in yeast, however, the transformants do not produce blue colour in the presence of Xgal.
D. When the linearized pGV256-dead is introduced into the yeast cells together with a linear DNA encompassing the region where 'STOP' codons have been inserted, the plasmid can be propagated either via integration of the linear PCR product (comprising 800 bp region of wild-type LacZ) into the linearized plasmid by homologous recombination (left hand side), in which case the transformants will produce blue colour in the presence of Xgal, or the plasmid will be re-ligated and the transformants will give white colonies.

severely debilitated with respect to the wild-type strain. In accordance with our *in vivo* LacZ-based recombination assay, the *yc1d* mutant strain exhibited reduction in survival rate on galactose-containing medium and only 60% of transformants were capable of surviving under galactose induction. Importantly, this reduction was restored to wild-type levels upon expressing a plasmid encoding Yc1dp in the *yc1d* mutant strain.

Discussion

Repair of the genotoxic lesions is critically important for prevention of genomic instability. Previous work from a number of laboratories has suggested that the nuclear matrix protein C1D could be involved in maintenance of genomic integrity by regulating activity of proteins involved in DSB repair. The identification of a family of C1D-related proteins from wide range of organisms (Fig. 1) tends to support the notion that C1D has an important biological function. In an attempt to understand the precise biological functions of C1D, we used *S. cerevisiae* as a model organism and analysed the direct consequences of loss of a functional C1D protein.

In agreement with the proposed role for human C1D, the *yc1d* mutant strain is sensitive for growth at 39°C, which is a common phenotype of mutant strains defective in preservation of genomic integrity (Boulton and Jackson, 1996b; Hryciw *et al.*, 2002; Peggie *et al.*, 2002). Moreover, the *yc1d* mutant strains, like those defective in *YKU70,* were found to be insensitive to DNA damaging agents such as MMS or UV, supporting the results obtained for human C1D, but surprisingly was only mildly sensitive to γ-irradiation. A mild sensitivity to γ-irradiation and resistance to other DNA damaging agents appears to be a phenotype shared among a restricted subset of genes. A genome-wide screen of diploid mutants homozygous for deletions of 3670 non-essential genes revealed 107 new loci that have an effect on γ-sensitivity (Bennett *et al.*, 2001). Interestingly, although about 90% of these were sensitive also to other DNA damaging agents, a group of diploid mutants containing 14 newly identified genes exhibited resistance to all DNA damaging agents but γ-irradiation.

The sensitivity of the *yc1d* mutant strain to high temperatures and slightly to γ-irradiation was highly reminiscent of Yku70p and prompted us to examine the possibility that *YC1D* might play a role in DSB repair. As DSBs can be repaired both by NHEJ and HR, the effects of deletion of *YC1D* was analysed for both pathways. Using the plasmid-based DNA repair assay, we demonstrated that the *yc1d* mutant strains are defective in DNA-end joining to a similar extent as the *yku70* mutant strain. The only difference between the *yc1d* and *yku70* mutant strains was in the efficiency of repair of the 5′-overhanging ends.

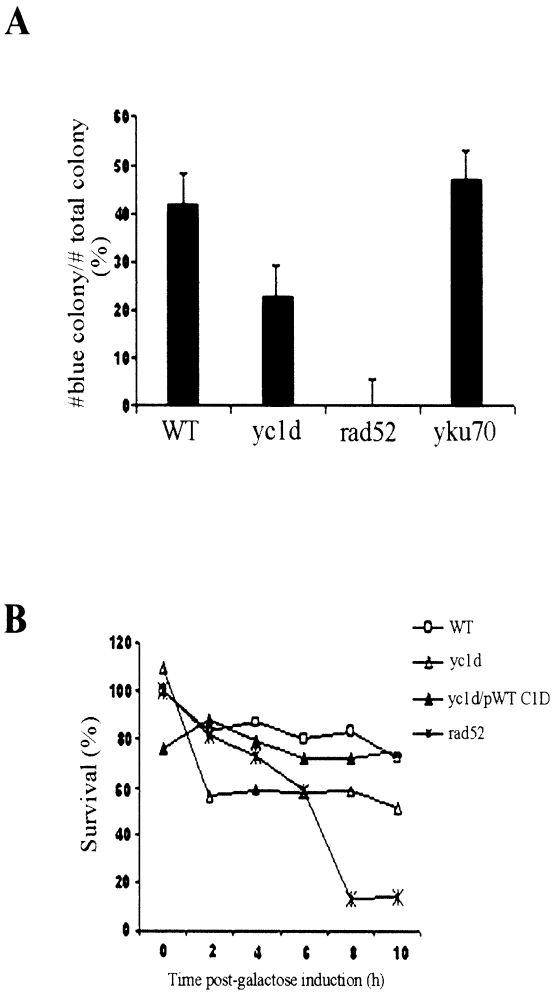

Fig. 7. The *yc1d* mutant strain is deficient in homologous recombination (HR).
A. The pGV256-dead was linearized from the unique *Bgl*II site and introduced into the indicated yeast strains together with a PCR fragment encompassing the region of LacZ where the STOP codons were integrated. Colony lift β-galactosidase assay was performed and the ratio of the number of blue colonies to the total number of colonies was calculated.
B. Survival of strains containing a plasmid expressing a galactose-inducible homothallic switching (HO) endonuclease expressed relative to survival at time zero.

Although still defective with respect to the wild-type strains, the *yc1d* mutant strain was slightly more efficient than *yku70* in repairing the 5′-overhanging DNA ends, suggesting that the role of Yc1dp in NHEJ is mainly in the 3′-end processing, an essential step in appropriate joining of DNA ends. Moreover, our work has also revealed that in the absence of Yc1dp, around 50% of 3′-overhanging ends are repaired inaccurately, indicating that another role of Yc1dp in NHEJ is to suppress the error-prone double-strand break repair pathways. By inhibiting this pathway, Yc1dp may contribute to maintaining genome integrity by

reducing the possibility of repair-associated mutations being introduced into the DNA.

To identify the function of Yc1dp in HR, a novel *in vivo* recombination assay was developed. This assay is simple to apply and rapid with respect to the other conventional assays used to measure the HR efficiency. The validity of this novel assay was confirmed by using a *rad52* mutant strain that is defective in HR and a *yku70* mutant strain that behaves like a wild-type strain. Using this assay, a *yc1d* mutant strain was analysed for efficiency of HR. The *yc1d* mutant strain exhibited a twofold reduction in HR efficiency with respect to the wild-type strain. Although this was not as dramatic as the defect in a *rad52* mutant strain, it nevertheless suggested that Yc1dp has a role in the HR pathway. More importantly, these results were also confirmed using a conventional HO endonuclease assay, which measures the HR efficiency in yeast cells. In accordance with the LacZ-based recombination assay, in the *yc1d* mutant strain, only 60% of the colonies harbouring an inducible plasmid expressing HO endonuclease were capable of surviving on galactose-containing medium, suggesting that *yc1d* mutant strains are defective in HR. In addition, when a plasmid expressing wild-type C1D was introduced into the *yc1d* mutant strain, the HR efficiency was restored back to the wild-type levels. In conclusion, the results obtained on *YC1D* so far demonstrate that *yc1d* mutant strains show sensitivity to increased temperature and γ-irradiation and that Yc1dp functions in NHEJ and, in contrast with Yku70p, Yc1dp is also required for efficient HR.

Interestingly, when a *yc1d/yku70* mutant strain was analysed for NHEJ, it was demonstrated that, the double knockouts were even less efficient in DNA 3′-end-joining with respect to the *yc1d* or *yku70* single mutant strains, suggesting that Yku70p and Yc1dp co-operate in this process. Although we cannot exclude the possibility that this collaboration may be through direct interaction of Yku70p and Yc1dp proteins, studies performed in mammalian systems demonstrated that the human C1D and Ku70 do not interact directly (Yavuzer *et al*., 1998) but rather that C1D interacts with the kinase subunit of DNA-PK. DNA-PK together with ataxia telangiectasia-mutated (ATM) and AT-related (ATR) proteins are important in DNA damage responses in higher eukaryotes (Smith and Jackson, 1999). In *S. cerevisiae*, a homologue for the catalytic subunit of DNA-PK is not found, however, the ATM/ATR homologues, Tel1p and Mec1p, are also important in DNA damage signalling (Weinert, 1998). Whether Yc1dp can also interact with yeast factors such as Tel1p and Mec1p that may play a similar role to DNA-PK is not known.

Two main repair pathways in eukaryotes, HR and NHEJ, begin in the same way; the ends of the DSB are resected by 5′- to 3′-exonucleases or by a helicase coupled to an endonuclease to produce 3′-overhanging DNA tails (Haber, 2000a). Therefore, processing of broken DNA ends is critical for repair of DNA-DSBs, and absence of proteins involved in this process can affect the efficiency of DNA-DSB repair pathways. It was reported that in eukaryotes, Ku70 acts as a switch between NHEJ and HR repair pathways and induces NHEJ while repressing HR (Goedecke *et al*., 1999; Pierce *et al*., 2001). As previously shown in humans and yeast, Rad50, Mre11 and Xrs2 (Nbs1 in humans) (RMX) complex is involved in processing of broken DNA ends (Haber 2000a; b). Interestingly, genetic studies have implicated the role of the RMX gene products in the same NHEJ pathway as Yku70p and Yku80p (Boulton and Jackson, 1998; Critchlow and Jackson, 1998). In addition to playing a major role in NHEJ, the RMX complex is also involved in HR, telomere maintenance, mating type switching and meiotic recombination. More recently, this complex has also been implicated in the suppression of chromosomal rearrangements (Chen and Kolodner, 1999) and in cell cycle checkpoint signalling (D'Amours and Jackson, 2001; Grenon *et al*., 2001; Usui and Schiebel, 2001). These findings suggest that the biological role of the RMX complex is in maintenance of genomic integrity. Similar to the RMX complex, Yc1dp has dual function in both HR and NHEJ repair pathways, which raises the possibility that Yc1dp could also have a role(s) in other RMX complex-related functions. Indeed, this suggestion coincides with our previous results that imply a role for human C1D in preservation of genomic integrity by regulating factors involved in DNA repair and recombination. Studies into a possible interaction between Yc1dp and the RMX complex proteins would be of great importance to decipher the molecular mechanism that *YC1D* uses while performing its function.

Experimental procedures

Plasmids and clonings

All yeast–*Escherichia coli* shuttle vectors used in this study have OriC sequences, an auxotrophic yeast selectable marker and a β-lactamase gene for ampicillin selection in *E. coli*. Plasmids used are as follows: pGV255-live (provided by L. Guarente) and pRS313 (Stratagene) were used for plasmid-based *in vivo* non-homologous DNA end joining (NHEJ) assays (yeast selectable markers are *URA3* and *HIS3* respectively). pCHOL (kindly provided by Allison Rattray, Fred Hutchinson Cancer Research Center, USA) was used for homothallic switching (HO) endonuclease assays. The pGV256-dead that was used for *in vivo* homologous recombination (HR) assay was constructed as follows: the oligonucleotide, 5′-CTGACTGAGTGAAGATCTTCACTCAGTCAG GAGCT-3′, was designed as a self-annealing primer and a *Sac*I restriction half-site was placed at the 3′-end. To obtain a double-stranded oligonucleotide, 10 ng of primer was incubated at 95°C for 2 min, 70°C for 10 min, followed by at 37°C for 10 min and, finally, incubated at room temperature for an additional 10 min. The double-stranded oligonucleotide was

then cloned into the pGV255-live, which was linearized from the unique *Sac*I site within the LacZ coding sequences. The bacterial colonies harbouring the pGV256-dead were selected by plating the transformants onto Luria–Bertani (LB)-plates containing Xgal (25 μg ml^{-1}) and IPTG (0.1 mM), and white colonies were selected. The plasmid DNA isolated from these colonies was confirmed by restriction enzyme digest.

Yeast strains

Apart from the *yc1d/yku70* double mutant (constructed for this study) and *mec1–21* mutant strains (kindly provided by Jessica Downs, Wellcome/CRC Institute, Cambridge, UK), all the strains used in this study were purchased from EUROSCARF. FY1679 and FY1679–08A are the wild-type diploid and haploid strains. Y01909 (Mat-a) and Y11909 (Mat-α) are the haploid strains mutant for *YHR081W* (*YC1D*). Y21909 and Y31909 are the heterozygote and homozygote diploid mutant strains for *YHR081W*. Y00870 and Y00540 are haploid mutant strains for *YKU70* and *RAD52* respectively. Polymerase chain reaction (PCR)-mediated gene knockout strategy was applied to delete *YC1D* on a *yku70* mutant background. The oligonucleotide primers used for this purpose had 5′ regions (≈ 40 bp) homologous to a region 200 bp upstream (forward primer) or downstream (reverse primer) of the *YC1D* open reading frame (ORF). The 3′-ends of the primers contained a 20 bp region that shows homology to *HIS3* gene. The sequences of the primers are as follows: CAAAGCGGCAACGTCATAACCTTGGTATTTATTGGGCAA CGTTTTAAGAGCTTGGTGAGC and CAAAAGTGTTCACT GCCAACTACAAGAATAGCATATACACATTCGAGTTCAAGA GAAAAA. The disruption cassette obtained via PCR was transformed into the Y00870 (*yku70* mutant strain) using the lithium acetate method, plated onto His$^-$ plates, and the His$^+$ colonies were obtained. The DNA was isolated and integration of the disruption cassette was confirmed via PCR.

Yeast media, growth conditions and transformation

Non-selective (YPAD) and selective (YC) media was used (Sherman *et al*., 1979). Mid-log phase liquid yeast cultures were prepared by inoculating one large colony per 5 ml of medium and incubating the culture at 30°C for 16–18 h with shaking at 230–270 r.p.m. The culture was then diluted in TE five times by serial 10-fold dilutions. Aliquots (5 μl) of each dilution were spotted on YPAD plates with (0.01%) or without methyl methane sulphate (MMS) and were then incubated at 30°C for 3–4 days to analyse sensitivity towards MMS. To analyse temperature sensitivity of yeast strains, serial dilutions of yeast strains spotted on YPAD plates were incubated either at 25°C or 39°C for 3 days. For UV- and γ-irradiation sensitivity, serial dilutions of yeast strains spotted on YPAD plates in duplicates were exposed to either UV- irradiation at 100 j/m^2 (UV Stratalinker 1800, Stratagene) or 20 Gy of gamma (Cs^{137}) source, followed by incubation at 30°C for 3–4 days. The photos of the plates were captured with Bio-Rad MULTI ANALYST software. Transformation into yeast strains were performed by the lithium acetate method (Sherman *et al*., 1979).

In vivo *plasmid-based repair assay*

In total, 3 μg of *Saccharomyces cerevisiae–E. coli* shuttle plasmids, pRS313 or pGV255-live, were linearized with different restriction endonucleases (10 units from each) to produce 5′- (*Bam*HI or *Xho*I digests) and 3′-overhanging (*Sac*I and *Pst*I digests) or blunt ends (*Sma*I digest). Linearized plasmid was then purified from the agarose gel using gel extraction kit (Macherey–Nagel) and 100 ng of the linearized and supercoiled versions of the plasmids were transformed into yeast strains in parallel, and the transformants were plated onto histidine-deficient YC agar plates followed by incubation at 30°C for 3–4 days until yeast colonies appeared. The colonies on each plate were then counted and normalized to the number of colonies obtained by the supercoiled version of the same plasmid. For NHEJ accuracy assays, *Sac*I-linearized and supercoiled forms of pGV255-live were used.

In vivo *plasmid-based homologous recombination assay*

For homologous recombination assay, 5 μg of pGV256-dead plasmid was linearized with *Bgl*II and was purified from the agarose gel by gel extraction kit. A 800 bp region of LacZ coding sequences encompassing the region where the 'STOP' codons were integrated was amplified by PCR from the pGV256-live plasmid, using the oligonucleotide primers LacZ forward (AGACGGATCCTCCTTTGCGAATACGCCCA C) and LacZ reverse (AGACGAATT**C**TGTGAAAGAAAGCC TGACTG). Amplified LacZ fragment (10 ng) was transformed into the recipient yeast strain together with the *Bgl*II-digested linear pGV256-dead plasmid (90 ng) by lithium acetate method. Transformed yeast cells were plated on uracil-deficient YC agar plates and incubated at 30°C for 3–4 days. After, the colonies appeared, colony-lift β-galactosidase assay using Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was performed and the number of colonies was counted. The ratio of blue colonies over to the total number of colonies on a selective plate was taken as the efficiency of homologous recombination for those particular yeast strains.

Colony-lift β-galactosidase assay

Yeast colonies were transferred onto nitrocellulose filter (Hybond C, Amersham). The filter was then placed, colony side up, in an aluminium boat floating in liquid nitrogen, and was immersed into the liquid nitrogen for 30 s. The filter was then transferred to a Petri dish containing one Whatmann filter circle soaked in 1.5 ml Z′ buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 2.7 μl ml^{-1} of β-mercaptoethanol) and 150 μl of Xgal (50 mg ml^{-1}). The dish was covered and plates were incubated in a 30°C incubator for 3–4 h, until the blue colonies appeared.

HO endonuclease sensitivity assay

The pCHOL, which expresses HO endonuclease under the control of *GAL I* promoter, was transformed into the appropriate yeast strains and the transformants were grown to mid-

log phase (OD_{600} = 0.5–0.8), washed twice with ddH_2O and resuspended in leu-minus minimal liquid medium containing either 2% glucose or galactose as carbon source. Samples were taken at various time-points, the number of cells was determined by measuring at OD_{600} and cells were plated on non-selective YPAD plates. The survival rate was calculated as the proportion of colony-forming units (cfu) to total cells and was normalized to the number of colonies before galactose induction.

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